Thyroid Hormone Regulation of Gene Expression in the Developing Rat Fetal Cerebral Cortex: Prominent Role of the Ca^{2+}/Calmodulin-Dependent Protein Kinase IV Pathway

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Thyroid hormones influence brain development through regulation of gene expression mediated by nuclear receptors. Nuclear receptor concentration increases rapidly in the human fetus during the second trimester, a period of high sensitivity of the brain to thyroid hormones. In the rat, the equivalent period is the last quarter of pregnancy. However, little is known about thyroid hormone action in the fetal brain, and in rodents, most thyroid hormone-regulated genes have been identified during the postnatal period. To identify potential targets of thyroid hormone in the fetal brain, we induced maternal and fetal hypothyroidism by maternal thyroidectomy followed by antithyroid drug (2-mercapto-1-methylimidazole) treatment. Microarray analysis identified differentially expressed genes in the cerebral cortex of hypothyroid fetuses on d 21 after conception. Gene function analysis revealed genes involved in the biogenesis of the cytoskeleton, neuronal migration and growth, and branching of neurites. Twenty percent of the differentially expressed genes were related to each other centered on the Ca^{2+} and calmodulin-activated kinase (Camk4) pathway. *Camk4* was regulated directly by T_3 in primary cultured neurons from fetal cortex, and the Camk4 protein was also induced by thyroid hormone. No differentially expressed genes were recovered when euthyroid fetuses from hypothyroid mothers were compared with fetuses from normal mothers. Although the results do not rule out a specific contribution from the mother, especially at earlier stages of pregnancy, they indicate that the main regulators of thyroid hormone-dependent, fetal brain gene expression near term are the fetal thyroid hormones. (***Endocrinology*** **151: 810–820, 2010)**

Thyroid hormones are extremely important for mammalian brain development and maturation. They control a wide range of developmental processes, such as neuronal and glial cell differentiation and migration, axonal myelination, and synaptogenesis. Accordingly, situations leading to thyroid hormone deficiency during human development may cause irreversible mental retardation and variable degrees of neurological impairment (1, 2). The effects of thyroid hormones are largely mediated through the control of gene expression. The period of maximal sensitivity of the rat brain to thyroid hormones spans the first 2–3 wk after birth, and genes related to neuronal migration, differentiation, and myelination are targets of thyroid hormones during this period (1, 3). The equivalent developmental period in humans extends from birth up to 1–2 yr of age.

There is also clinical and epidemiological evidence supporting an earlier role of thyroid hormones in fetal brain

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Abbreviations: Camk4, Ca^{2+}/calmodulin-activated kinase IV; CR, Cajal-Retzius cells; Creb1, cAMP-responsive element-binding protein 1; CT, calcitonin; E13, embryonic d 13; MMI, 2-mercapto-1-methylimidazole; NS, not significant; Tx, thyroidectomized.

development. For example, the classical syndrome of neurological cretinism and the consequences of maternal hypothyroidism and hypothyroxinemia on the fetal brain are thought to be due to thyroid hormone deficiency before midgestation, most likely during the second trimester (4–6). The T_3 receptor is already expressed in the human brain by the beginning of the second trimester of gestation (7), before onset of fetal thyroid gland secretion, which occurs around the 20th week of pregnancy. At these developmental stages, increasing type 2 deiodinase activity favors the selective accumulation of T_3 in the developing cerebral cortex (7, 8).

The second trimester of human pregnancy is roughly equivalent to the second half of gestation in rodents (9–11). In the rat, the nuclear receptor protein concentration in the fetal brain is undetectable before embryonic d 13 (E13) and then increases reaching about 30% of adult values by E17 (12). These values are maintained throughout the rest of the fetal period and increase subsequently after birth. Thyroid hormone content and secretion by the fetal thyroid gland, and T_4 and T_3 concentrations in plasma and brain are very low before E19 and increase severalfold in brain up to term pregnancy. By E21, 58% of adult concentrations of T_4 and T_3 are present in the fetal brain (13). Before onset of thyroid gland function, maternal thyroid hormones contribute significantly to the fetal pool, and even minor and transient deficiencies of T_4 in the mother cause altered cerebral cortex development in the fetus (14). Despite these observations, the rat fetal cerebellum at term was found unresponsive to thyroid hormones in terms of gene expression (15). This is conceptually in disagreement with the above evidence. Although the developmental timing of cerebral and cerebellar cortices is different, it raises the possibility that the consequences of thyroid hormone deprivation for fetal brain development might be due to indirect causes unrelated to the genomic actions of thyroid hormone. Further efforts to identify genes regulated by thyroid hormones in the fetal brain have met with limited success (16, 17).

To understand the role of thyroid hormones on early brain development, and to dissect the relative roles played by the fetal and maternal hormones, knowledge of their molecular targets in the fetal brain are needed. We have analyzed the effects of maternal and fetal hypothyroidism on global gene expression in the cerebral cortex of rat fetuses at term pregnancy. We tried to answer the two following questions. Do thyroid hormones influence gene expression in the fetal cortex? And, what are the relative roles of the maternal and fetal hormones? Our data represent the first systematic effort to identify molecular targets of thyroid hormone in the fetal brain, specifically in the developing neocortex. We found that changes of gene expression were not due to a nonspecific effect of maternal hypothyroidism on the fetal brain, and rather, they were correlated with the fetal thyroid hormones. The regulated genes were present in networks involved in nervous system development and function, particularly influencing cell to cell signaling and interaction, and cell movement and maturation. In particular, the Ca^{2+}/calmodulin-activated kinase IV (Camk4)-cAMP-responsive element-binding protein 1 (Creb1) pathway seems to play an important role in thyroid hormone-dependent fetal cerebral cortex development.

Materials and Methods

Animal handling

Young adult female Wistar rats grown in our animal facilities and weighing 250–300 g were used. Protocols for animal handling were approved by the local institutional Animal Care Committee and followed the rules of the European Union. Animals were under temperature-controlled (22 ± 2 C) and light-controlled (12-h light, 12-h dark cycle; lights on at 0700 h) conditions and had free access to food and water. All surgical interventions were under anesthesia by inhalation of 1.5–2% isoflurane (Esteve, Barcelona, Spain) in O_2 (0.9 liters O_2/min).

To induce maternal and fetal hypothyroidism, the pregnant dams were thyroidectomized (Tx) on E10 (the day of appearance of the vaginal plug was E0) and given 0.02% 2-mercapto-1-methylimidazole (MMI) (Sigma Chemical Co., St. Louis, MO) in the drinking water until they were killed. To induce isolated maternal hypothyroidism, the pregnant dams were Tx on E10 and given tap water as previously described (18). From the day of thyroidectomy until killing, the pregnant dams were infused with rat PTH (19) (PTH 1-84; Bachem H3086; Bachem, Torrance, CA), 4 μg/100 g body weight · d, and rat calcitonin (CT) (20) (Bachem H3072), 1 μg/100 g body weight · /d as described (21). Both PTH and CT were diluted in 0.1 M acetate buffer (pH 4.0) and simultaneously infused using osmotic minipumps placed under the dorsal skin with a delivery ratio of 1 μl/h · d (Alzet model 2001, www.alzet.com, Cupertino, CA). The Tx rats were also supplemented with 0.16% Ibercal-D (Merck S. L., Madrid, Spain), which contains vitamin D3 (9.5 IU/100 ml drinking water) and calcium pidolate (11.8 mg/100 ml). This treatment prevented hypocalcemia and ensured a normal number of fetuses per litter (21). The dams of the control group were sham operated and implanted with osmotic pumps containing solvent. Serum PTH, CT, and calcium were measured as described (21) in the rats used for microarray hybridizations at the end of the experiment on E21 (see below). Control (n = 10) and Tx+MMI (n = 10) dams had similar concentrations (mean ± SD) of circulating CT [control = 0.13 ± 0.08 ng/ml; Tx+MMI = 0.18 ± 0.08 ng/ml, not significant (NS)], PTH (control = 57.4 ± 16.3 pg/ml; Tx+MMI = 66.9 ± 9.7 pg/ml, NS), and total calcium (control = 9.44 ± 0.91 mg/100 ml; Tx+MMI = 8.70 ± 1.16 mg/100 ml, NS). The brains of the pups were carefully removed and the cerebral cortices dissected and immediately frozen in liquid nitrogen. Thyroid hormones and TSH were measured as described (22).

Microarray hybridization

Total RNA was isolated from the cerebral cortex of individual pups. The Trizol procedure (Invitrogen, Carlsbad, CA) was followed, with an additional step of chloroform extraction. DNA from the same samples was used to determine the sex of the pups by PCR amplification of the *Sry* gene using as sense primer 5′-CAGAGATCAGCAAGCATCTGG-3′ and antisense primer 5′-TCTGGTTCTTGGAGGACTGG-3′ (23). The quality of RNA was analyzed using a BioAnalyzer (Agilent, Santa Clara, CA). Equal amounts of RNA from three pups of the same sex from each litter were pooled, and 250 ng RNA was used as template for the cDNA synthesis with the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). The microarray assays were done using the Agilent platform (Agilent whole rat genome array, 60-mer 4 × 44 slide format). These arrays contain 41,012 probes representing 14,209 unique genes and 20,061 nonmapped probes/expressed sequence tags (based on annotations downloaded from NCBI on September 2, 2008).

Analysis of the data from microarray hybridizations

Analysis for differential expression was performed using the R platform for statistical analysis (24) and several packages from the Bioconductor project (25). The raw data were imported into R and preprocessed using the half method for background correction and the quantile method for normalization. Probes with expression level below the detection control probe in all samples were removed. To identify differentially expressed genes, we used the limma package (26) that fits a gene-wise linear model, allowing us to account for different experimental and technical factors. Experimental factors included sex and treatment and a batch effect to account for technical variation. Correction for multiple testing was accomplished by controlling the false discovery rate using the method from Benjamini and Hochberg (27).

Pathway analysis

The Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com) was used to identify the biological functions and/or diseases that were most significantly represented in the data set. The program uses the Ingenuity's knowledge database containing large amounts of individually modeled relationships between genes obtained from the literature. Data containing differentially expressed genes and the corresponding expression values were uploaded into the application. Interactions between molecules of interest with other molecules in the database were identified as network eligible molecules (focus genes) and used to construct functional regulatory networks. Networks of these focus genes were then algorithmically generated based on their connectivity. Networks are scored based on the number of focus genes they contain. The network score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's exact test. The score is the negative log of this *P* value. The higher the score, the lower is the probability of finding the observed number of focus genes in a given network by random chance.

Functional analysis was performed on these networks to identify the biological functions and/or diseases that were most significant to the genes in the network. Fisher's exact test was used to calculate a *P* value to determine the probability that each biological function and/or disease assigned to that network is due to chance alone.

We also performed a promoter analysis using the BiblioSphere Pathway Edition version 7.20 (Genomatix Software, Munich, Germany; http://www.genomatix.de). This software integrates literature mining (from published abstracts) and annotation analysis (Gene Ontology). In addition, genes found to be co-cited with a transcription factor are analyzed with Genomatix MathInspector for transcription factor binding sites in the promoters of the co-cited genes (28).

Primary cultures

All media were purchased from Invitrogen. The cerebral cortices were dissected from E17 normal rat fetuses in Hanks' balanced sodium salt solution (HBSS), without Ca^{2+} and Mg^{2+}, supplemented with 1 mM sodium pyruvate and 10 mM HEPES (pH 7.4). The tissue was disaggregated by passing through a 0.9-mm syringe, rinsed in HBSS/pyruvate/HEPES, and resuspended in serum-free culture medium (neurobasal medium supplemented with 2% B27, 0.5 mM glutamine, 10 U/ml penicillin, and 10 U/ml streptomycin), before seeding on poly-L-ornithine-coated 12-well multiwells (Sigma) (2.5×10^5 cells per well). After 4 d, the cells were incubated 24 or 48 h in the same medium without B27 supplement before adding 5 nM T_3 (Sigma). Control cultures without T_3 added were incubated in parallel. To examine the response to T_3 in the presence of inhibition of protein synthesis, cycloheximide (Sigma) was added to the cultures at a final concentration of 8 μg/ml 30 min before T_3, and the cells were harvested 6 h after T_3 addition. All assays were run in triplicate.

Western blotting

Proteins were extracted from neuronal cultures in 0.15 M NaCl, 10% glycerol, 0.0015 M $MgCl_2$, 0.001 M EDTA, 0.1 M NaF, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 0.05 M HEPES (pH 7.4) to which the following protease inhibitors were added: 0.1 M Na_3VO_4, 0.025 M β-glycerol-P, 0.01 mg/ml leupeptin, 0.01 mg/ml aprotinin, and 0.001 M phenylmethylsulfonyl fluoride. Thirty micrograms of each sample were run in 10% acrylamide gels. For Western blotting, we used anti-Camk4 antibody (1:1000; Sigma) and antirabbit IgG (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA) for secondary antibody; anti-β-tubulin (1:5000; Abcam, Cambridge, MA), and antimouse IgG (1:5000, Santa Cruz). The blots were developed in 0.275 mM pcumaric acid, 1.25 mM luminol, 36% H_2O_2, 0.1 M Tris-HCl (pH 8.8).

Real-time PCR

Real-time PCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems). A cDNA aliquot corresponding to 5 ng of the starting RNA was used. Assay-on Demand TaqMan primers and TaqMan Universal PCR Master Mix, No AmpErase UNG, were purchased from Applied Biosystems. The PCR program consisted in a hot start of 95 C for 10 min, followed by 40 cycles of 15 sec at 95 C and 1 min at 60 C. Assays were performed in triplicate, using the 18S gene as internal standard and the 2^{-Ct} method for analysis.

For biological confirmation of the microarray results, we used RNA from pups of different litters from those used for microarray hybridization. As starting material for these experiments, we used pooled RNA from three fetuses of each litter and compared the pools of different litters. In addition, some probes were con-

firmed by analyzing a large number of RNA samples of individual fetuses from several litters. For analysis of gene expression in cultured cells, we used RNA isolated from about 5 million cells in triplicate. Significance of differences between experimental groups was calculated by the Student's *t* test when two groups were compared. When comparing more than two groups, we performed one-way ANOVA and the Tukey *post hoc* test (GraphPad Software, San Diego, CA; www.graphpad.com).

Results

Models of maternal and fetal hypothyroidism

We validated the models of maternal and fetal hypothyroidism used in this work by measuring TSH in the maternal and fetal serum and T_4 and T_3 in the maternal serum and the fetal cortex (Fig. 1). For these determinations, we used a different group of animals from those used for microarray analysis, but similarly treated. Tx dams showed increased TSH and decreased T_4 and T_3, as expected. However, the fetuses from Tx rats had normal serum TSH and cerebral cortex T_4 and T_3. Tx dams treated with MMI showed similar changes in maternal serum as Tx. The pups from these rats were also hypothyroid, with elevated TSH and decreased cortex T_4 and T_3 (Fig. 1). Therefore, thyroidectomy of the dams resulted in maternal hypothyroidism, whereas additional treatment with MMI resulted in combined maternal and fetal hypothyroidism.

Changes in gene expression in the fetal cerebral cortex induced by maternal and fetal hypothyroidism

To identify candidate genes regulated by thyroid hormones in the fetal cerebral cortex, we first used the model

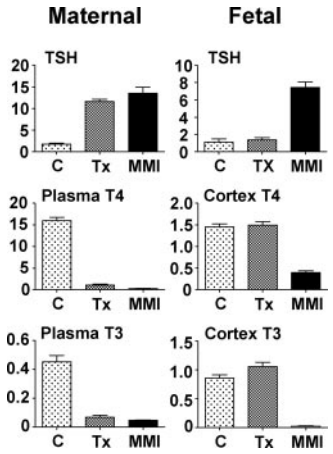

FIG. 1. Models of maternal and fetal hypothyroidism: concentrations of serum TSH in the maternal and fetal compartments and of T_4 and T_3 in the maternal serum and fetal brain. C, Control dams; MMI, Tx+MMI dams. Number of animals was 5 for each value. One-way ANOVA indicated significant differences ($P < 0.001$) between C and either Tx or MMI for the maternal values and between MMI and either C or Tx for the fetal values. All other comparisons were NS. All units are nanograms per milliliter.

of combined maternal and fetal hypothyroidism. Maternal serum TSH, T_4, and T_3 were again measured in this group as an added control for this experiment. T_4 in the control dams (n = 17) was 18.2 ± 7.4 ng/ml and was reduced by about 95% in the hypothyroid dams (T_4 = 0.8 ± 0.1 ng/ml, $P < 0.0001$; n = 17). Serum T_3 in the control dams was 0.416 ± 0.109 ng/ml and was reduced by about 87% in the hypothyroid dams (0.035 ± 0.003 ng/ml, $P < 0.0001$). Circulating TSH in the control dams was 4.73 ± 0.89 ng/ml and was increased 6-fold in the hypothyroid dams (28.16 ± 5.3 ng/ml, $P < 0.0001$). From this group, 10 control and 10 hypothyroid litters of similar size (between nine and 12 pups) were selected.

For microarray hybridizations, we started from RNA pools of three males from each of five control and five hypothyroid litters and of three females from an equal number of different litters. In a previous work on the effect of thyroid hormone in the developing postnatal cerebellum (29), it was shown that differential gene expression was influenced by sex. Therefore, to analyze a possible effect of sex in the expression of genes in the fetal cerebral cortex, we added the sex factor, in addition to treatment, to the statistical model. The resulting distribution of *P* values was flat, and only a few probes were expressed differently in males compared with females. Most of them correspond to genes linked to the sex chromosomes (see supplemental Data 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org), and there was no difference in expression of these genes between hypothyroid and control fetuses (supplemental Table 1). Therefore, for statistical analysis of the arrays, we used all the data from the 10 control and 10 hypothyroid litters, without further taking into account the sex of the pups.

Using a statistical cutoff of $P_{adjust} < 0.01$, we obtained 1252 differentially expressed probes (552 unique genes), of which 583 probes (244 genes) were up-regulated and 669 (308 genes) down-regulated by hypothyroidism (the complete list is provided in supplemental Table 1). From this list, 428 genes were identified in the Ingenuity knowledge database and used to construct functional networks. The detailed list of individual genes included in these networks is provided in supplemental Table 2. The biological functions assigned to the most significant networks correspond to cell-to-cell signaling and interaction, cellular assembly and organization, cellular function, nervous system development and function, cellular movement, and organ development. A detailed analysis for some functional subcategories revealed genes involved in the biogenesis of cytoskeleton, migration, growth, and branching of neurites (Table 1).

A prominent role of the Camk4 and Creb1 pathway was suggested by the finding that 56 genes contained

TABLE 1. Significant processes affected by fetal hypothyroidism in the cerebral cortex

Function annotation	*P* value	Molecules	No. of molecules
Biogenesis of cytoskeleton	8.40×10^{3}	CALR, CNN1, CSRP1, DYNLL1, KRAS, NEFH, NEFL, NEFM, PACSIN2, PAFAH1B1	10
Quantity of filaments	2.50×10^{4}	FMOD, FN1, KRAS, NEFH, NEFL, NEFM, PAFAH1B1, SERPINH1, TPM1	9
Assembly of neurofilaments	2.52×10^{5}	NEFH, NEFL, NEFM	3
Clustering of neurofilaments	2.52×10^{5}	NEFH, NEFL, NEFM	3
Organization of neurofilaments	1.26×10^{3}	NEFH, NEFL, NEFM	3
Dissociation of neurofilaments	2.52×10^{5}	NEFH, NEFL, NEFM	3
Accumulation of neurofilaments	9.86×10^{5}	NEFH, NEFL, NEFM	3
Size of axons	9.86×10^{5}	NEFH, NEFL, NEFM	3
Arborization of dendrites	1.26×10^{3}	NEFH, NEFL, NEFM	3
Branching of neurites	1.65×10^{3}	KLF9, NEFH, NEFL, NEFM, NOS1, SLIT1, SLIT2	7
Bifurcation of dendrites	2.94×10^{2}	NOS1	1
Growth of neurites	9.40×10^{4}	CREB1, FN1, HAP1, KRAS, MAPK1, SLIT1, SLIT2	7
Cell movement of normal cells	2.20×10^{2}	C2, CALR, CCND1, CD86, CTSS, FLT1, FN1, GCNT1, GNAS, HIST1H1T, HRH3, HSPD1, IFNAR1, IL12A, ITGAE, LCP2, LTB, MADCAM1, MAPK1, NOV, PAFAH1B1, PNOC, RGS3, SLIT2, TGFB2	25
Cell movement of neurons	1.72×10^{2}	PAFAH1B1, RGS3, SLIT2	3
Positioning of cells	2.31×10^{4}	LTB, PAFAH1B1, PVRL2, SLIT1, SLIT2	5
Positioning of neurons	5.79×10^{3}	PAFAH1B1, SLIT1, SLIT2	3
Guidance of axons	5.12×10^{3}	ANK3, ARX, CHN1 (includes EG:108699), CNTN4, MAPK1, SEMA3B, SLIT1, SLIT2, TGFB2	9
Guidance of thalamocortical axons	4.97×10^{3}	SLIT1, SLIT2	2
Chemorepulsion	1.06×10^{2}	FLT1, SEMA3B, SLIT2	3
Migration of neural crest cells	2.69×10^{2}	ACAN, FN1, SEMA3C, SLIT2	4
Recognition of neurons	8.12×10^{3}	OPCML, SLIT2	2
Stratification of cerebral cortex	8.61×10^{4}	DAB1, PAFAH1B1	2
Proliferation of cerebral cortex cells	8.02×10^{4}	ADCYAP1R1, CREB1	2
Development of cortical plate	2.94×10^{2}	DAB1	1
Depolarization of neurons	7.20×10^{3}	ADCYAP1R1, CACNG8, NTS	3
Depolarization of pyramidal neurons	2.94×10^{2}	CACNG8	1
Transport of endosomes	1.98×10^{2}	HAP1, SNX16, SQSTM1	3
Formation of clathrin-coated pits	2.53×10^{3}	ANXA6, PICALM	2
Exocytosis	1.36×10^{2}	ATP2A2, EXOC7, HRH3, RPH3AL, SNAP23, SYT2, SYTL5, VAMP4	8
Function of organ	2.90×10^{3}	CALR, CREM, FLT1, GREM1, HOMER1, SOSTDC1, TXNIP	7
Binding of cells	1.76×10^{2}	ADAM15, ADAM1A, ADIPOR2, CALR, CD86, CNTNAP1, F3, FN1, GCNT1, GNAS, GPC1, HAS2, HIST1H1T, HS3ST1, ITGAE, LCP2, RGS3, SEMA3B, SERPINF1, SERPINF2, TGFB2	21
Invasion of cells	1.06×10^{2}	CALR, CCND1, ENTPD5, EZH2, FBLN5, FLT1, FN1, GPI, HAS2, JUNB, KLF4, MAPK1, MYCN, NOV, ODC1, PICALM, SERPINF2, SLC9A3R1, SLIT2, SPDEF, WISP1	21
Neurological process of mice	4.14×10^{3}	ADCY8, ADRBK2, AGA, CALB1, CANX, CREB1, CREM, DBP, DYRK1A, GNB1 liter, GRK5, HOMER1, HSD11B2, NOS1, NTS, PNOC	16
Long-term memory of rats	4.97×10^{3}	CREB1, CREM	2
Circadian rhythm	4.89×10^{3}	CREB1, CREM, DBP, HTR7, JUNB, MAPK1	6
Analgesia of mice	1.38×10^{2}	ADCY8, ADRBK2, NOS1, NTS	4
Neurotransmission	2.96×10^{5}	HAP1, HOMER1, HTR7, MAPK1, PAFAH1B1, PNOC, ROM1, SLC17A7	8
Neurogenesis	5.46×10^{4}	CNTN4, CREB1, DYRK1A, HAP1, PAFAH1B1, SLIT1, SLIT2, TGFB2	8

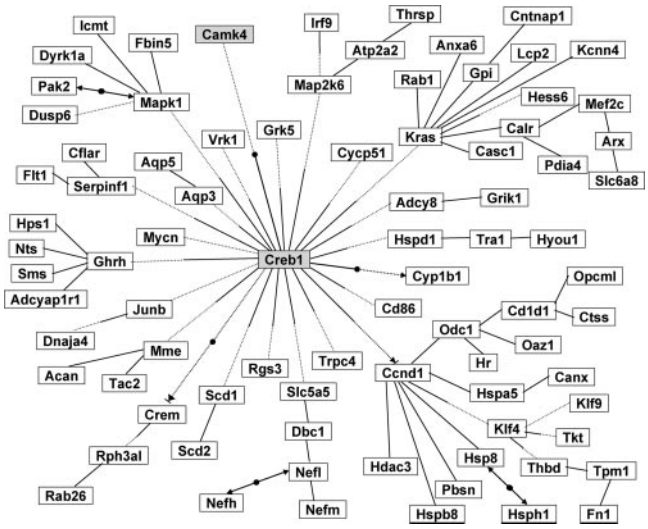

FIG. 2. Interrelations of differentially expressed genes centered on Camk4-Creb1. This analysis was performed using the BiblioSphere Pathway Edition version 7.20 (Genomatix Software). The analysis integrates literature mining (from published abstracts) and annotation analysis (Gene Ontology). Genes found to be co-cited with a transcription factor were analyzed using Genomatix MathInspector for transcription factor binding sites in the promoters of the co-cited genes. Those co-citations verified as functional (such as "gene A … <function word> … gene B") by inspection of the pertinent PubMed abstracts were represented by connection lines between the genes. *Arrowheads* at the end of a *connecting line* symbolize the type of functional relationship between the connected genes (*arrowheads* indicate activation, and *blocked arrowheads* indicate inhibition). The *dotted line* indicates that a gene encoding a transcription factor is connected to a gene known to contain a binding site for this transcription factor in its promoter. Hand-annotated gene-gene relationships are indicated by a circle in the center of the connection line.

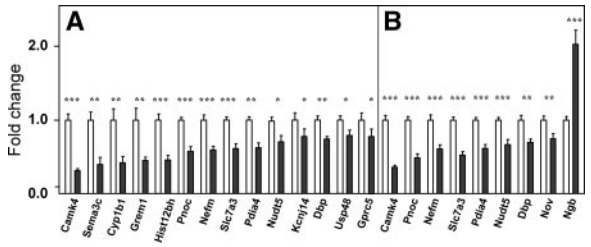

FIG. 3. Confirmation by real-time PCR of the effect of hypothyroidism on gene expression in the fetal cortex. Expression data were normalized by giving a value of 1 to the mean value of controls. A, Assays were done in pools from three fetuses of each litter. Six control litters (*white bars*) and seven hypothyroid litters (*black bars*) were analyzed for each gene target. B, Assays were done with individual fetuses, using six fetuses per litter. Four control litters (24 individual samples) and three hypothyroid litters (18 individual samples) were analyzed for each target gene. Significance of differences was calculated by the Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P \leq 0.001$.

Creb1-binding sites in their promoter region (supplemental Table 3). BiblioSphere Pathway analysis disclosed that 82 genes (about 15% of all differentially expressed genes) were related to each other centered on Camk4/Creb1. Figure 2 shows these relationships, by integrating published data on genes regulated directly or indirectly by the transcription factor Creb1, which is activated by Camk4-induced phosphorylation.

PCR confirmation

Real-time PCR was used for confirmation of gene expression changes induced by hypothyroidism. These experiments were performed in biological replicates, *i.e.* in a different group of pups than those used for the arrays but obtained from similarly treated dams. We selected the target genes on the basis of several criteria, namely their relative abundance, expression changes above 1.6-fold, known involvement in developmental processes, and availability of predesigned TaqMan probes. We followed two approaches, with similar results. In the first approach, six control and seven hypothyroid litters were individually analyzed using cortex RNA pools from three randomly selected pups of each litter (Fig. 3A). In this approach, individual variability among the fetuses of the same litter was averaged by analyzing a single pool from each litter. Figure 3B shows the results of PCR using an alternative approach with cortex RNA from individual fetuses. For this analysis, we employed six fetuses from each of four control litters and six fetuses from three hypothyroid litters, making a total of 24 control and 18 hypothyroid fetuses. Sixteen genes of 20 were confirmed as being differentially expressed in the hypothyroid fetuses, with the largest effect found on *Camk4*. Differences in expression of four genes (three from the up-regulated set and one from the down-regulated set) were not significant, although the mean fold changes were in the same direction as in the arrays. Mean fold changes ± SE for these four genes were as follows: *Prom2*, 1.7 ± 0.3 ($P = 0.08$); *C8g*, 1.6 ± 0.4 ($P = 0.21$); *Cirbp*, 1.2 ± 0.2 ($P = 0.34$); and *Hs3st1*, 0.8 ± 0.1 ($P = 0.21$).

Relative roles of the maternal and fetal thyroid glands

The effects of hypothyroidism on fetal gene expression, as analyzed with the previous experimental model of combined maternal and fetal hypothyroidism, might be a direct consequence of thyroid hormone action or secondary to other metabolic alterations induced by maternal and/or fetal hypothyroidism. It was also possible that the replacement treatment with CT and PTH might have influenced the expression of sensitive genes. To check the effect of these factors, we analyzed gene expression in the cerebral cortex of euthyroid fetuses from Tx mothers. Tx of the dams was performed on E10. Circulating T_4 in the Tx dams of this group was one order of magnitude lower than in the control dams (1.6 ± 0.5 *vs.* 16.4 ± 3.8 ng/ml, $P < 0.0001$). Microarray hybridizations were performed by comparing E21 fetal cerebral cortices derived from 12 control dams and 12 hypothyroid dams. Analysis of the

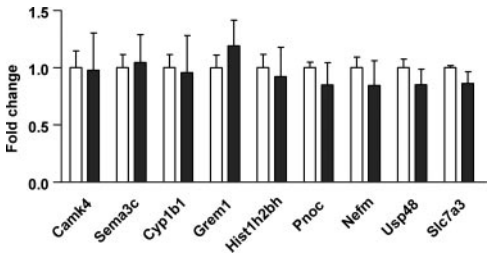

FIG. 4. Lack of effect of maternal Tx on gene expression in the fetal cerebral cortex on E21. Fetuses were obtained from Tx dams, so that the fetuses remained euthyroid, whereas the mother was hypothyroid. The RNA substrate for PCR was prepared from pools of three fetuses from the same litter. Litters from five control (*white bars*) and five Tx (*black bars*) dams were analyzed.

microarray data revealed that there were no changes of gene expression below a significance level of $P < 0.05$ (not shown). To substantiate the lack of effect of isolated maternal hypothyroidism, we measured the expression of nine of the genes shown above to be most sensitive to maternal and fetal hypothyroidism (Fig 4). There was no change in expression of these sensitive genes, indicating that in the presence of maternal hypothyroidism, the fetal hormones secreted during the last days of gestation are able to maintain thyroid hormone-dependent cerebral cortex gene expression within normal levels.

Direct effect of T_3 on primary cultures of fetal cortex neurons

To examine the direct responsiveness to T_3, we used primary neuronal cultures obtained from the cerebral cortex of normal E17 fetuses. The cells were incubated in the presence or absence of T_3 to measure the expression of the same group of 16 genes shown in Fig. 3 (Fig. 5). In preliminary experiments 5 and 50 nM T_3 gave similar results 24 h after addition to the cultures, so that in subsequent experiments, we used 5 nM T_3. A significant response was found for *Camk4*, *Nefm*, *Sema3c*, *Grem1*, *Pnoc*, *Nov*, *Slc7a3*, and *Gprc5* (Fig. 5, *upper panel*). No effect of T_3 was found on *Dbp*, *Pdia4*, *Nudt5*, *Cyp1b1*, *Kcnj14*, *Hist1h2bh*, *Usp48*, and *Ngb*, although the change for the latter was in the expected direction. In another experiment, using a different preparation of cells, we checked the response at 24 and 48 h after T_3 addition. The effect on *Camk4*, *Grem1*, *Nefm*, and *Nov* was highest at 48 h. For *Pnoc* and *Slc7a3*, the maximal effect was obtained at 24 h (Fig. 5, *lower panel*).

Addition of T_3 to the neurons in primary culture also induced the accumulation of the Camk4 protein, with a maximal effect at the highest time point studied (72 h, Fig. 6, *upper panel*). Finally, to analyze whether the effects of T_3 were direct or required the synthesis of intermediate proteins, we analyzed the effect of T_3 6 h after addition in the presence or the absence of cycloheximide (Fig. 6, *lower panel*). Of the genes showing a clear effect of T_3 in the primary cultures, only *Camk4* was induced in the presence of cycloheximide. *Camk4* expression was increased significantly by T_3 already 6 h after addition to the cultures. Cycloheximide had a mRNA-stabilizing effect, because Camk4 mRNA was also increased in the presence of this drug. However, cycloheximide did not prevent a further increase when T_3 was also added. For other genes, as shown for *Nefm*, no effect of T_3 was observed at 6 h, and therefore it was

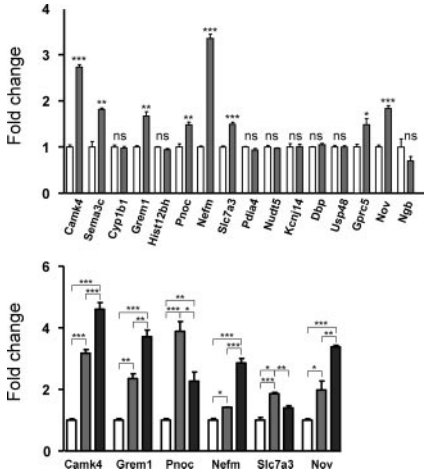

FIG. 5. Stimulation of gene expression by T_3 in primary neuronal cultures from E17 fetal cerebral cortex. T_3 (5 nM) was added to the cultures, and expression of the indicated genes was measured by real-time PCR 24 h (*gray bars*) and 48 h (*black bars*) after T_3 addition. The values are expressed as fold change over controls, giving a value of 1 to the cells incubated in the absence of T_3. One-way ANOVA with Tukey test for all comparisons: *, $P < 0.05$; **, $P < 0.01$; ***, $P \leq 0.001$.

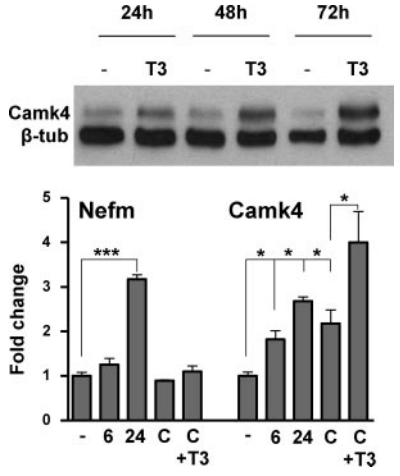

FIG. 6. *Upper panel*, Stimulation of Camk4 protein synthesis at different times after addition of 5 nM T_3 to the cultures. Control marker is β-tubulin (β-tub). *Lower panel*, Effect of cycloheximide on Nefm and Camk4 mRNA induction by T_3. Cells were incubated with T_3 for 6 or 24 h with T_3, as indicated, or 6 h with cycloheximide alone (C) or with T_3 (C + T_3). One-way ANOVA: *, $P < 0.05$; ***, $P \leq 0.001$.

not possible to analyze the effect of cycloheximide. Longer incubation times in the presence of cycloheximide were not tested.

Discussion

Previous studies on thyroid hormone action in the fetal brain

Despite extensive work on the actions of thyroid hormones in brain development there are still many uncertainties as to how thyroid hormones influence maturation of the fetal brain, and to related questions such as the relative roles of the maternal and fetal thyroids, or the timing of action of thyroid hormones. One of the difficulties in approaching these problems is the almost complete lack of knowledge on the molecular targets of thyroid hormones in the developing fetal brain. Some years ago it was concluded that the fetal rat brain is not sensitive to thyroid hormones in terms of gene expression (15). However, the patterns of T_3 accumulation, and of T_3 receptor and deiodinase expression in the human and rodent brain suggest otherwise. During the second trimester of human pregnancy there is a selective accumulation of T_3 in the cerebral cortex. There are also many clinical and epidemiological data indicating that thyroid hormones exert important role in brain maturation before mid gestation in humans (corresponding to the end of the fetal period in the rat) as evidenced by neurological cretinism and related syndromes (2, 9).

Previous studies have identified target genes of thyroid hormones during the postnatal period in the rat (1) or in adult animals (31), but studies focused on the fetal brain have led to only fragmentary information. In fact, studies using models of maternal and fetal hypothyroidism as employed in this work have identified genes that were sensitive to thyroid hormones in the postnatal brain but insensitive in the late fetal brain (32). Other studies have shown quantitative changes in specific mRNAs or proteins after maternal hypothyroidism or thyroid hormone treatment (17), but a global analysis of the effect of thyroid hormones on the fetal brain has not been made.

Limitations of the microarray approach

It is often pointed out that a major difficulty in studies of gene expression in the brain is the high cellular and molecular complexity of brain tissue, precluding the recovery of cell type-specific information. A recent analysis on the human brain transcriptome suggests that it is possible to detect significant expression levels of genes with restricted expression in specific cells using total brain RNA (33). In our study, we detected the expression of genes of restricted expression in the layer I-specific, Cajal-Retzius (CR) cells, such as *Ebf3* (CR over non-CR expression = 2342/9) (34), indicating that the use of a complex tissue such as the cerebral cortex was not a major impediment to isolate thyroid hormone-sensitive genes. A different but related question is whether it is possible to detect cell-restricted changes of expression of genes being expressed in different cell types. Thus, we did not recover *Rln* as a differentially expressed gene, as reported previously (35), suggesting that nonregulated expression in non-CR cells masked the changes in the CR cells (*Rln* expression in the CR cells over non-CR cells = 15,546/979) (34). Therefore, the methodology used in this work allowed the identification of genes regulated by thyroid hormones throughout the cortex or in major cell populations but had limitations to recover cell-specific responses. This limitation should be kept in mind when analyzing the possible contribution of the maternal hormones to gene expression in the fetal brain.

Correlation between morphological alterations of hypothyroidism and significantly affected pathways

We have identified a relatively large number of genes of altered expression in the fetal cerebral cortex as a consequence of hypothyroidism. Ingenuity pathway analysis disclosed that nervous system development and function, cell to cell signaling, and posttranslational modifications were among the pathways significantly affected. More specifically, among the relevant functions affecting the development of the cerebral cortex were those related to cell movement and positioning, assembly of neurofilaments, and branching of neural processes. Although this conclusion falls within what should be expected from the morphological effects of hypothyroidism, the data provide a molecular framework for the known effects of thyroid hormone on cell migration, cerebral cortex layering, and branching of dendrites.

Significance of Camk4 induction

We found a prominent role for the Camk4-Creb1 pathway and downstream targets (36) in the action of thyroid hormone in the fetal cortex. Camk4 was previously shown to be induced by T_3 in rat fetal telencephalic cultures (37, 38) and in mouse embryonic stem cells (39). Developmental expression of *Camk4* correlates with periods of neuronal differentiation and apoptosis (40) and has been implicated in brain-derived neurotrophic factor (BDNF) action (41). Induction of Camk4 and Creb1 downstream targets may represent an important process in thyroid hor-

mone action in the fetal cortex, because it should facilitate Ca^{2+} regulation of dendritic growth (42). The transcriptional activity of the nuclear T_3 receptor is enhanced by Camk4 (44). Therefore, induction of Camk4 may potentiate T_3 signaling, a process that might be highly relevant in the developing brain, at stages when the concentrations of T_3 and its nuclear receptor are still low. Potentiation of T_3 receptor signaling by Camk4 may be due to direct phosphorylation of coactivators (44) or by disrupting apo-receptor repression through the silencing mediator for retinoic acid and thyroid hormone (SMRT) corepressor and histone deacetylases. This latter mechanism has been demonstrated after synaptic activity-dependent Ca^{2+} activation of Camk4 (45) and may potentiate thyroid hormone-dependent stimulation of synaptogenesis. Based on these data, we propose that Camk4 regulation is an essential part of an amplifying mechanism for T_3 action in the fetal brain at stages where both the concentration of T_3 and that of receptor are still low. In support of this proposal, a thyroid hormone-responsive element has been identified in the *Camk4* gene promoter (39), and as we show here, the stimulation by T_3 in cultured neurons was direct.

Lack of effect of maternal hypothyroidism

It might be argued that the genomic effects observed as a consequence of hypothyroidism in the fetal brain are nonspecific consequences of hypothyroidism, especially of the mother, and would not reflect a direct disruption of thyroid hormone signaling. Direct actions of T_3 are supported by its effects on primary cultured neurons, and at least one of the gene targets, *Camk4*, was regulated directly at the transcriptional level. The nonresponsive genes might represent downstream actions of T_3 or permissive actions of the hormone requiring cooperation with additional factors present *in vivo* and absent from the cultures. In addition, we did not find significant changes of gene expression in the cerebral cortex of euthyroid fetuses born from Tx mothers. In this experiment, the fetuses had grown in a hypothyroid milieu from E10 to at least the onset of fetal thyroid secretion on E17–E18. The subsequent accumulation of T_3 in the fetal brain was effective in maintaining normal gene expression on E21. Therefore, we may conclude that changes of gene expression in the hypothyroid fetal cortex are not due to general nonspecific effects of hypothyroidism. This conclusion agrees with previous data showing that the metabolic effects of maternal thyroidectomy during the second half of gestation are compensated by a normally functioning fetal thyroid gland (46).

Relative roles of the maternal and the fetal thyroid hormones

The fact that fetuses from Tx mothers showed no changes in cortex gene expression suggests that the fetal thyroid hormones are the main regulators of fetal brain gene expression at term. This is not to say that maternal hypothyroidism is irrelevant in gestation. It is known that deficient contribution of maternal hormones to the fetus is compensated by increased fetal thyroid secretion (13). In line with this, TSH has been reported to be increased in fetuses from hypothyroid dams at term gestation (46, 47), although other authors found that it was unchanged (30, 43). We found that fetuses from Tx dams had normal serum TSH and brain T_4 and T_3 concentrations on E21, indicating efficient compensation by the fetal thyroid gland. Despite these observations, maternal hypothyroidism from E16 to birth induced by thyroidectomy leads to structural defects of the cerebral cortex and the hippocampus of the offspring (21). The molecular basis for these alterations remains unknown and may consist of subtle actions, probably restricted to specific cellular groups. Maternal thyroid hormones should also be important at earlier stages of gestation and especially before onset of fetal thyroid hormone secretion (16, 17) or in situations of isolated fetal hypothyroidism. The experimental approach and the molecular targets described in this paper are valuable tools to investigate this issue in more detail.

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